Inoculum effects of cefepime/zidebactam (WCK 5222) and ertapenem/zidebactam (WCK 6777) for Enterobacterales in relation to β-lactamase type and enhancer effect, as tested by BSAC agar dilution

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Running head: Inoculum effects for zidebactam combinations

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Abstract

Introduction. Combinations of PBP3-active β-lactams with developmental diazabicyclooctanes (DBOs), e.g. zidebactam, remain active against many metallo-β-lactamase (MBL) producers, via an enhancer effect. We explored how this was affected by inoculum.

Materials and Methods. MICs of zidebactam and its cefepime and ertapenem combinations (WCK 5222 and WCK 6777, respectively) were determined by BSAC agar dilution at inocula from 3-6 x 10^3 to 3-6 x 10^5 cfu/spot. Isolates, principally *Klebsiella* spp., were chosen as having previously tested resistant to zidebactam or its cefepime combination, and by β-lactamase type.

Results. MICs of zidebactam, tested alone, were strongly inoculum dependent, regardless of β-lactamase type; MICs of its cefepime and ertapenem combinations likewise were strongly inoculum dependent – rising ≥32-fold across the inoculum range tested – but only for MBL producers. Combination MICs for isolates with non-metallo-β-lactamases, including those with OXA-48 (where the enhancer effect remains critical for ertapenem/zidebactam), were much less inoculum dependent, particularly for cefepime/zidebactam. MBL producers frequently moved between putative 'susceptible' (MIC ≤8+8 mg/L) and 'resistant' (MIC >8+8 mg/L) categories according to whether the inoculum was at the high or low end of the BSAC's acceptable (1 to 4 x 10^4 cfu/spot) range.

Conclusion. The activity of zidebactam combinations against MBL producers – which strongly depends on the enhancer effect – is inoculum dependent. Animal data suggest consistent *in-vivo* activity even in high inoculum pneumonia models. Contingent on this being supported by clinical experience, the combination behaviour may be best represented by the MICs obtained at the lower end of BSAC's inoculum range.

Introduction

Combinations of β-lactams with 'triple action' diazabicyclooctanes (DBOs) – zidebactam, nacubactam or durlobactam – provide a prospective option against carbapenemase-producing Gram-negatives, regardless of whether these have class A, B or D β-lactamases.[1] The furthest-progressed combination, cefepime/zidebactam (WCK 5222), is entering phase III, whilst nacubactam combinations are at an earlier stage, as is ertapenem/zidebactam (WCK 6777), envisaged as a once-daily product suitable for OPAT.

The distinguishing feature of these developmental DBOs is that they not only inhibit serine β-lactamases, as does avibactam (the parent DBO), but that they additionally bind to PBP2, achieving a direct antibiotic activity and, when partnered with a PBP3-targetted β-lactam, a β-lactamase-independent synergy dubbed the 'enhancer effect'.[2-4] These secondary properties allow their combinations with PBP3-targetted β-lactams to remain active against many isolates with Class B (i.e., metallo) carbapenemases, although these are not inhibited by any DBO.[2] The enhancer effect is important also for ertapenem/zidebactam in the case of isolates with OXA-48 carbapenemase, which is not inhibited by zidebactam,[2] though not for cefepime/zidebactam, which incorporates a cephalosporin stable to OXA-48 enzyme that needs only be protected against co-produced ESBLs.

A limitation is that high-frequency mutations, largely affecting the stringent response, can compensate for inhibition of PBP2, conferring zidebactam and nacubactam resistance.[5] Even then, the enhancer effect persists, and β-lactam/zidebactam or β-lactam/nacubactam combinations remain active against many zidebactam- and nacubactam-resistant metallo-β-lactamase (MBL) producers.[2-4] Thus, multiple large surveys, variously by broth microdilution or agar dilution, find that 90-95% of MBL-producing Enterobacterales are susceptible to cefepime/zidebactam 8+8 mg/L;[6-9] though, using agar dilution, we recorded a lower proportion

(45%) among a small collection (n=24) of *Klebsiella* spp. with both NDM and OXA-48 β-lactamases, many belonging to ST14.[9]

What remains unclear is the extent to which the enhancer activity is affected by inoculum, particularly when zidebactam itself lacks activity. To explore this aspect we compared MICs of zidebactam, cefepime/zidebactam and ertapenem/zidebactam at inocula from *c.* 3-6 x 10^3 to 3-6 x 10^5 cfu per spot for zidebactam-resistant MBL producers, where activity depends on the enhancer effect, and zidebactam-resistant producers of zidebactam-inhibited serine β-lactamases, where the enhancer effect has secondary importance.

Method and Materials

Bacteria

A panel of 93 isolates, encompassing three groups, was assembled from an earlier study,[9] where testing also was performed by BSAC agar dilution with cefepime/zidebactam as a 1:1 gravimetric ratio. Group 1 comprised 33 isolates previously found resistant to cefepime/zidebactam 8+8 mg/L; it included 32 *K. pneumoniae* and one *Escherichia coli* with NDM carbapenemases alone (n=20) or together with OXA-48 (n=13). Group 2 comprised 30 MBL producers previously found highly resistant (MIC ≥32 mg/L) to zidebactam, but susceptible to cefepime/zidebactam at ≤8+8 mg/L. These included 28 *K. pneumoniae* and two *K. oxytoca* with carbapenemases as follows: IMP (n=5), VIM (n=6), NDM (n=17) and NDM plus OXA-48 (n=2). Group 3 comprised 30 MBL-negative isolates previously found highly resistant to zidebactam (MIC ≥32 mg/L) but susceptible to cefepime/zidebactam at 8+8 mg/L. They variously produced KPC (n=9), OXA-48 (n=9) or GES-5 (n=2) carbapenemases, K1 enzyme (n=1) and either ESBLs or AmpC enzymes alone or in combination (n=9); 25 were *K. pneumoniae*, 3 were *K. oxytoca* and 2 *K. aerogenes*.

MIC determinations.

The UK Health Security Agency's (UKHSA, formerly Public Health England, PHE) AMRHAI Reference Unit performed susceptibility tests by the BSAC agar dilution method,[10] adapted as below, using IsoSensitest agar from Oxoid/Thermofisher (Basingstoke, UK). Zidebactam combinations were tested as 1:1 ratios, with zidebactam and ertapenem from Wockhardt (Aurangabad, India) and cefepime purchased from Alfa Aesar (Heysham, UK).

Standard inocula were prepared by adjusting bacterial suspensions to match a 0.5 McFarland standard (*c.* 1-2 x 10^8 cfu/mL), then printing on to antibiotic-containing agars using the fine-pin inoculator (Mast, Merseyside, UK) which delivers 0.3 μl, thereby giving *c.* 3-6 x 10^4 cfu/spot inocula (see Discussion). In parallel, we performed MIC determinations with 0.3 (i.e., 3.3-fold) and 0.1 (i.e., 10-fold) dilutions of these standard suspensions, giving 1-2 x 10^4 cfu/spot and 3-6 x 10^3 cfu/spot, respectively, and with suspensions matched to 1.5 and 5 McFarlands, respectively delivering 3x and 10x standard inocula, corresponding to 1-2 x 10^5 cfu/spot and 3-6 x 10^5 cfu/spot.

Plates were read to where growth terminated: fine films and hazes were ignored, as was the presence of 1 or 2 isolated colonies. Trailing was mostly an issue when zidebactam was tested alone, particularly with lower inocula; on/off trailing/regrowth was ignored but persistent heavy trailing was counted as growth.

Results

Confirmation of prior results as a basis for Group assignment

Most isolates behaved in accordance with previous results at the standard inoculum, supporting their categorisation into Groups 1-3; disagreements largely were instances where cefepime/zidebactam MICs of 8+8 mg/L were recorded for Group 1 isolates previously found resistant with MICs one tube higher at 16+16 mg/L. For zidebactam, tested alone, 88/93 prior

and current results agreed within one doubling dilution, with larger discrepancies equally distributed between cases where the present result was >1 dilution higher (n=2) or lower (n=3). For cefepime/zidebactam, 77/93 isolates had prior and current results within one doubling dilution, again with larger discrepancies equally distributed between instances where the present result was >1 dilution higher (n=7) or lower (n=9); 90/93 of cefepime/zidebactam results were within 2 doubling dilutions of previous data.

Inoculum effects

The inocula spanned a 100-fold range, from 3-6 x 10^3 to 3-6 x 10^5 cfu/spot, extending 10-fold either side of PHE's 3-6 x 10^4 cfu/spot 'standard'. As anticipated, based upon how the panel was constructed, almost all (89/93) the isolates were highly resistant to zidebactam at the standard inoculum, with MICs >32 mg/L (Table 1). This proportion rose to 92/93 at 10x standard inoculum; by contrast, and crucially, the proportion with zidebactam MICs >32 mg/L shrank to 52/93 at 0.3x standard inoculum and to 30/93 at 0.1x standard. At this lowest inoculum, zidebactam alone at 8 mg/L (i.e., as present in a 1:1 cefepime/zidebactam combination with a putative 8+8 mg/L breakpoint) inhibited 18/33 Group 1 isolates, 22/30 Group 2 and 21/30 Group 3 (*p* 0.04 Chi-square test), albeit with a higher modal MIC (1 mg/L) for Group 1 than for Groups 2 and 3 (both 0.25 mg/L).

The MBL producers of *both* Groups 1 and 2 exhibited strong inoculum effects for both β-lactam/zidebactam combinations, as illustrated in Table 1 and in fig. 1, which depicts the proportions susceptible to cefepime/zidebactam 8+8 mg/L and ertapenem/zidebactam 8+8 mg/L at different inocula. Fully 25/33 (75%) of the 'cefepime/zidebactam-resistant' Group 1 isolates became susceptible to cefepime/zidebactam at $\leq$8+8 mg/L if the inoculum was lowered to 0.1x standard; on the other hand, 22/30 of the 'cefepime/zidebactam-susceptible' Group 2 isolates became resistant if the inoculum was raised to 10x standard. We conclude that the

158 distinction between Groups 1 and 2 isolates is not fundamental; rather it is that the shift to high
159 MICs occurs at slightly lower inocula in Group 1 (fig. 1).

160 Measured across the 3-6 x 10^3 to 3-6 x10^5 inoculum range, the MIC inoculum effects
161 for the MBL-producing Group 1 and 2 isolates typically were 32- to >128-fold (fig 2), with
162 low ratios seen only for the minority of MBL producers that remained highly resistant to
163 cefepime/zidebactam and ertapenem/zidebactam even at the lowest inocula, raising the
164 denominator for the ratio. By contrast, there were only *c.* 1-4-fold MIC effects for
165 cefepime/zidebactam in the case of the zidebactam-resistant MBL-negative Group 3 isolates.
166 Effects were more scattered for ertapenem/zidebactam, being 1- to 4-fold for 20/30 Group 3
167 isolates, including all those with GES carbapenemases, ESBLs or AmpC enzymes but 8-32-
168 fold for the remaining 10, comprising 4/9 with OXA-48 carbapenemase and 6/9 with KPC
169 carbapenemases.

170

171 **Discussion**

172 MBLs hydrolyse cefepime and ertapenem and are not inhibited by zidebactam or other
173 DBOs.[1,2,11] Consequently, any activity of zidebactam combinations against MBL producers
174 must depend on the antibiotic activity of zidebactam and/or the enhancer effect. This enhancer
175 action must also drive the activity of ertapenem/zidebactam against isolates with OXA-48
176 carbapenemase, given that zidebactam is a poor inhibitor of this enzyme.[2] We explored the
177 extent to which these activities were affected by the inoculum, and the implications for
178 susceptibility testing.

179 Regardless of whether the MBL-producing isolates had previously been found resistant
180 (Group 1) or susceptible (Group 2) to cefepime/zidebactam at the standard inoculum, we
181 observed strong, mostly >32-fold, inoculum-dependent MIC rises both for zidebactam alone
182 and for its combinations (Table 1 and fig 1). By contrast, effects were absent

(cefepime/zidebactam) or smaller (ertapenem/zidebactam) for Group 3 isolates, which were zidebactam-resistant, but lacked MBLs. The differing behaviour of the Group 3 isolates can largely be explained by their having zidebactam-inhibited β-lactamases, allowing a conventional mode of activity. The exception to this generalisation is the case of ertapenem/zidebactam against isolates with OXA-48 enzymes, which are not inhibited by zidebactam; inoculum effects for these were similarly minimal to those observed for isolates with KPC carbapenemases, which are inhibited by zidebactam.[2] This anomaly may simply reflect the fact that OXA-48 enzymes confer less *in-vitro* resistance to ertapenem than do MBLs, limiting the scope for an effect: at standard inocula 6/9 isolates with OXA-48 enzymes were inhibited by *unprotected* ertapenem at ≤8 mg/L compared with just 9/63 among MBL producers (not shown).

β-Lactamase-related inoculum effects are well-known in Gram-negative bacteria, occurring e.g. for weak-substrate oxyimino-cephalosporins in the case of ESBL producers, first-generation cephalosporins for isolates with classical TEM enzymes and widely, for penicillin/β–lactamase-inhibitor combinations.[12-14] In our experience, supported by the published literature, such effects only become substantial above inocula of 10^5.[12-14] The effects outlined here were manifest at lower inocula, particularly for the Group 1 isolates, overlapping into the inoculum ranges used for susceptibility testing. Effects in this range also occur with mecillinam which, like zidebactam, attacks PBP2.[15] Although synergistic mecillinam/β-lactam combinations reportedly evade this issue[16] they were not tested for isolates with challenging combinations of resistance mechanisms, as used here.

Even minor inoculum variances may have a significant practical effect on results for zidebactam combinations. The BSAC method,[10] used here and formerly by the AMRHAI Reference Unit (which has subsequently switched to EUCAST microbroth testing as its standard method) specifies adjusting a bacterial suspension to the opacity of a 0.5 McFarland

(*c.* 1-2 x 10^8 cfu/mL), diluting this 10-fold for Enterobacterales, then using multipoint pins that deposit 1-2 μL per spot, giving an inoculum of *c.* 1-4 x10^4 cfu/spot. Since AMRHAI used fine-pin multipoint inoculators, depositing only 0.3 μl/spot, we adapted the method to use the 0.5 McFarland-equivalent suspension without dilution, giving *c.* 3-6 x10^4 cfu/spot. Taking 4.24 x 10^4 cfu/spot as the geometric mid-point of this range equates to an inoculum on the upper edge of the BSAC specificiation or minimally above it, whilst the 0.3x dilution contains an inoculum (geometric mid-point 1.41 x 10^4 cfu/spot) around the lower edge. Both are 'technically acceptable, yet the former would lead to more isolates being scored as resistant (Fig. 1).

In the absence of clinical experience, it is not possible to say which inoculum is most representative of the patient, nor the extent to which this may vary between patients or infection sites. Nonetheless, there are some pointers from animal testing, in particular a high-inoculum (6.69 + 0.31 $\log_{10}$ cfu/lung) murine pneumonia model using humanised dosing.[17] This found the ertapenem/zidebactam combination effective in achieving 2-3 $\log_{10}$ reductions in lung count even for MBL producers with ertapenem/zidebactam MICs of 8+8 mg/L and zidebactam MICs >128 mg/L by CLSI broth microdilution with an inoculum of *c.* 1 x 10^4/well. The reductions in bacterial load were similar to those for infections established using zidebactam-resistant isolates with non-metallo-carbapenemases and lower MICs. This was despite the fact that, at an MIC of 8+8 mg/L, the ertapenem concentration exceeded the MIC for only *c.* 15-18% of the dosage interval.

Whilst it is difficult to translate these animal data directly to the present study, it does suggest retention of enhancer activity against organisms where strong inoculum effects would be predicted, and despite a heavy bacterial challenge. Accordingly, whilst further work is needed, there is reason for optimism that the higher MICs observed with slightly raised inocula are not predictive of clinical failure, and that results with inocula close to 1 x 10^4 – at the lower

end of the BSAC range and represented by the 0.3x standard here – may be the most representative.

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330 **Table 1.** MICs of zidebactam and its combinations for carbapenemase- and cephalosporinase- producers in relation to prior results with cefepime/zidebactam and inoculum
331

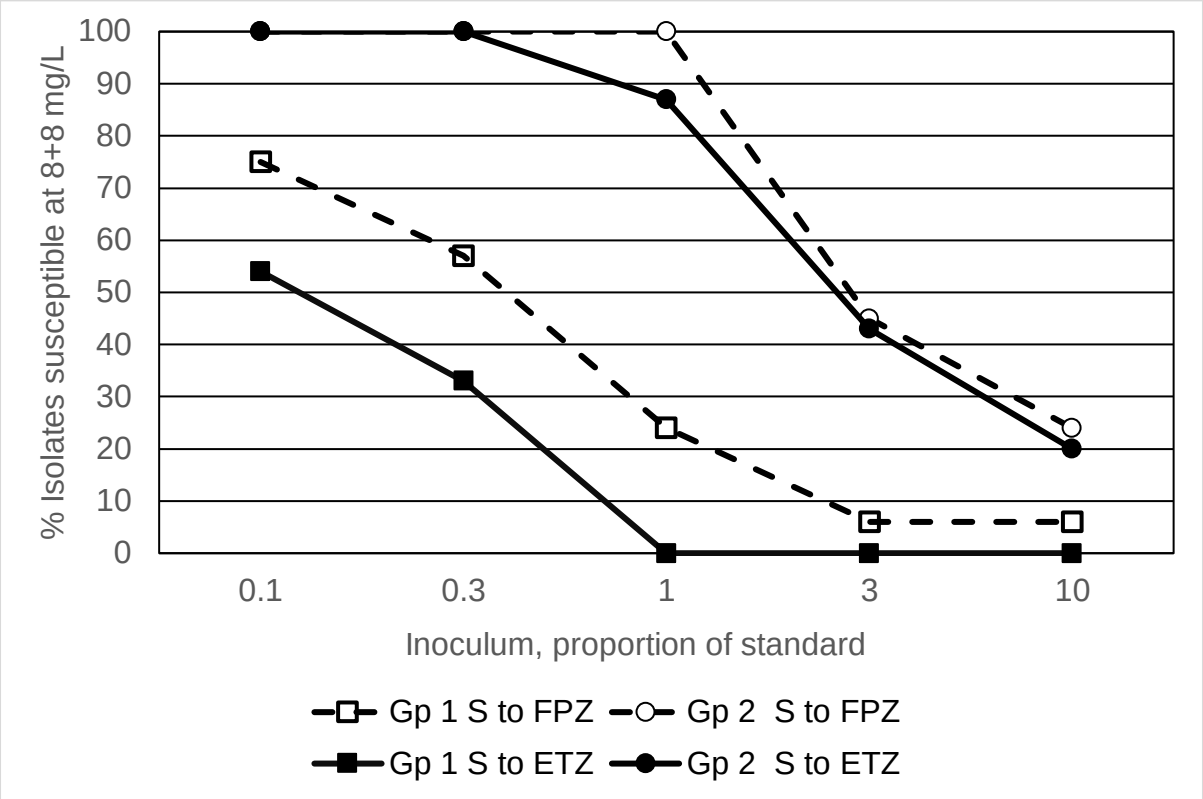
		No isolates with indicated MIC (mg/L)														
Drug and inoculum/spot	**Inoculum**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**>128**	**Total**
Zidebactam																
Group 1. MBL producers, previously found resistant to cefepime/zidebactam 8+8 mg/L	0.1 x standard					1	**10**	4	2	1		1		4	**10**	33
	0.3 x standard						5	4		2		5	2		**15**	33
	Previous distribution at standard inocula							1			1	1			**30**	33
	Standard										1			1	**31**	33
	3 x standard										1				**32**	33
	10 x standard										1				**32**	33
Group 2. MBL producers, previously found susceptible to cefepime/zidebactam 8+8 mg/L	0.1 x standard			3	**9**	4	3	3				1	1	2	**4**	30
	0.3 x standard			1	1	**4**	3	2				3	2	3	**11**	30
	Previous distribution at standard inocula											3	1	1	**25**	30
	Standard					1					1		1	1	**26**	30
	3 x standard														**30**	30
	10 x standard														**30**	30
Group 3. Non-metallo carbapenemase and cephalosporinase producers, previously found susceptible to cefepime/zidebactam 8+8 mg/L	0.1 x standard			1	**8**	6	5	1							**9**	30
	0.3 x standard				1	1	**5**				1	3	5	2	**12**	30
	Previous distribution at standard inocula												2	1	**27**	30
	Standard								1					1	**28**	30
	3 x standard														**30**	30
	10 x standard														**30**	30

		No isolates with indicated MIC (mg/L)														
Drug and inoculum/spot	**Inoculum**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**>128**	**Total**
Cefepime/zidebactam																
Group 1. MBL producers, previously found resistant to cefepime/zidebactam 8+8 mg/L	0.1 x standard					2	**16**	4	2	1	7			1		33
	0.3 x standard					1	**6**	5	3	4	**6**	4	3		1	33
	Previous distribution at standard inocula										**17**	12	2	2		33
	Standard					1	2			5	10	**11**	2	1	1	33
	3 x standard						1			1	1	1	10	**17**	2	33
	10 x standard						1			1		1	6	**17**	7	33
Group 2. MBL producers, previously found susceptible to cefepime/zidebactam 8+8 mg/L	0.1 x standard			8	**9**	8	2	3								30
	0.3 x standard			2	**8**	6	7	6		1						30
	Previous distribution at standard inocula				1	3	6	9	**11**							30
	Standard				2	3	**11**	4	5	5						30
	3 x standard				1		2	1	4	7	**9**	3	1	1	1	30
	10 x standard				1			1	2	4	5	**9**	6	1	1	30
Group 3. Non-metallo carbapenemase and cephalosporinase producers, previously found susceptible to cefepime/zidebactam 8+8 mg/L	0.1 x standard		2	8	**11**	5	3	1								30
	0.3 x standard			6	9	**11**	3	1								30
	Previous distribution at standard inocula			3	11	**12**	3	1								30
	Standard			2	9	**12**	5	2								30
	3 x standard			1	6	**12**	7	4								30
	10 x standard				8	**11**	7	4								30

		No isolates with indicated MIC (mg/L)														
Drug and inoculum/spot	**Inoculum**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**>128**	**Total**
Ertapenem/ zidebactam																
Group 1. MBL producers, previously found resistant to cefepime/zidebactam 8+8 mg/L	0.1 x standard					2	**11**	8	4	3	1	1	1	1	1	33
	0.3 x standard					1	5	**7**	4	6	1	1	**4**	3	1	33
	Previous distribution at standard inocula						1	1		2	2	6	**13**	7	1	33
	Standard						1	3		5	7	3	**8**		6	33
	3 x standard										2	2	4	12	**13**	33
	10 x standard									1	1		1	7	**23**	33
Group 2. MBL producers, previously found susceptible to cefepime/zidebactam 8+8 mg/L	0.1 x standard			**10**	2	**11**	3	4								30
	0.3 x standard			**7**	5	5	2	**6**	4	1						30
	Previous distribution at standard inocula			3	2	3	3	4	**7**	3	3	2				30
	Standard			3	3	4	2	**6**	2	**6**	3		1			30
	3 x standard				1	3		3	3	3	3	4	**7**	2	1	30
	10 x standard					1	2	2		1	4		4	**13**	3	30
Group 3. Non-metallo carbapenemase and cephalosporinase producers, previously found susceptible to cefepime/zidebactam 8+8 mg/L	0.1 x standard	2	1	3	**10**	8	2	4								30
	0.3 x standard		3	2	**9**	**9**	2	5								30
	Previous distribution at standard inocula	1	2	1	5	**12**	1	5	2	1						30
	Standard		2	2	4	**8**	7	4	2	1						30
	3 x standard		2	1	2	**7**	9	2	6		1					30
	10 x standard			3	1	4	5	**8**	5	3	1					30

Values in bold – modal values

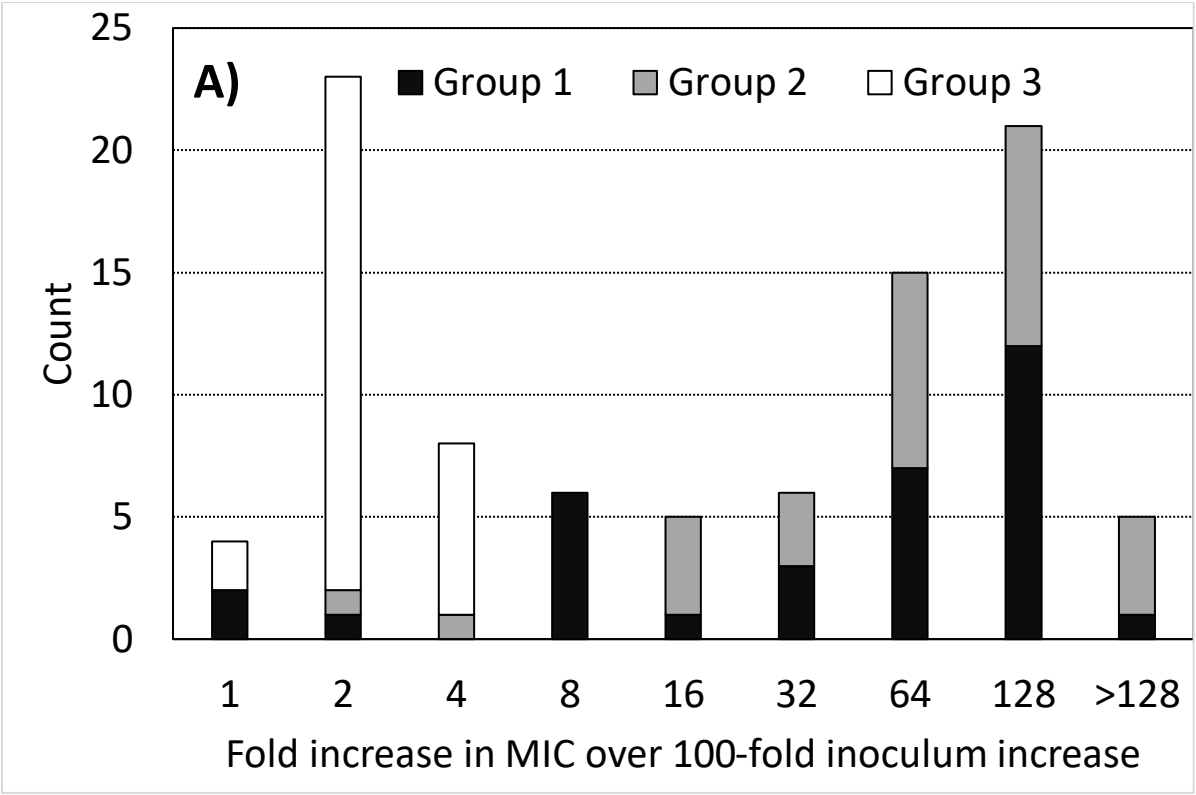
334 Figure 1
335

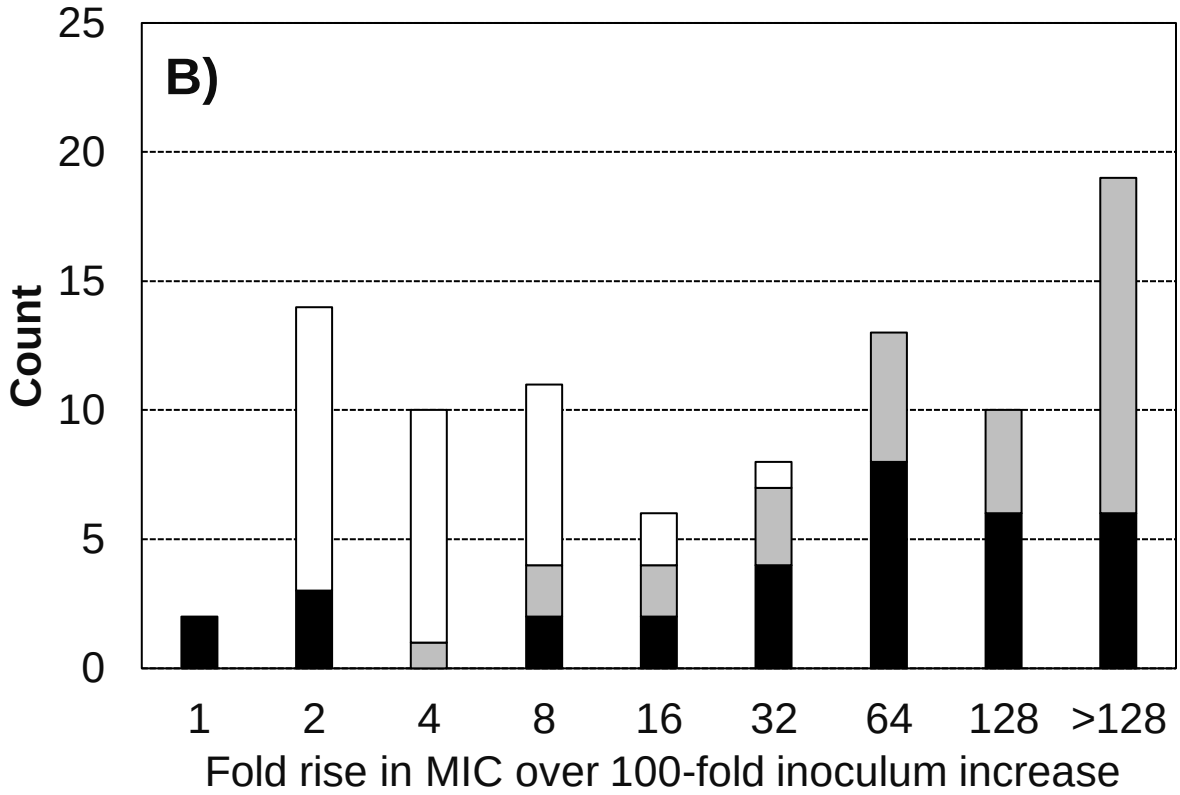


336

337

Figure 2


A)
Group 1
Group 2
Group 3
Count
25
20
15
10
5
0
1
2
4
8
16
32
64
128
>128
Fold increase in MIC over 100-fold inoculum increase


B)
Count
25
20
15
10
5
0
1
2
4
8
16
32
64
128
>128
Fold rise in MIC over 100-fold inoculum increase

Legend, Figure 1: Percent susceptibility to cefepime/zidebactam 8+8 mg/L (FPZ) and ertapenem/zidebactam 8+8 mg/L (ETZ) for Group (Gp) 1 and 2 isolates in relation to inoculum.

Legend, figure 2. Distribution of ratios of MICs at inocula of 3-6 x 10^5 vs. 3-6 x 10^3 for (top panel) cefepime/zidebactam 1:1 and (lower panel) ertapenem/zidebactam 1:1 by isolate group. The large inoculum effects for most Group 1 and 2 isolates are evident; the minorities of these groups with only small effects are cases where the low inoculum denominator MICs remained high, typically >128 mg/L (see Table 1).